HYDROGEN–TRITIUM EXCHANGE OF RHODOPSIN: EFFECT OF SOLVENT ON THE INCORPORATION OF SLOWLY EXCHANGING TRITIUM ATOMS

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1. Introduction

Rhodopsin, the visual pigment of the vertebrate retinal rod outer segments undergoes isomerisation of its chromophoretic group, retinal, on absorption of a photon. It has been widely suggested that after photoisomerisation of the retinal, rhodopsin is responsible for the release of a transmitter into the interdiscal space of the rod outer segment [1]. However this transmitter has not yet been identified; indeed it is not yet clear to what extent rhodopsin's polypeptide chain undergoes conformational changes upon photoisomerisation of the retinal.

The hydrogen–tritium exchange technique has often been used to demonstrate the presence of conformational changes in proteins [2,3]. They are visualised as changes in the exchange kinetics of the proteins labile hydrogens. However in order that a conformational change can be detected the labile protons of the polypeptide chain, associated with the conformational change considered, must have become tritiated during the exchange-in of incubation time. Parameters such as the pH, temperature, ionic strength, and the composition of the medium will change the freely accessible proton exchange rate [2] and/or the conformation of the protein and hence modify the observed exchange kinetics.

Thus in order that the conformational changes of rhodopsin upon illumination can be studied it is necessary to ensure that the associated labile hydrogens have become tritiated during the incubation time. The effect of several incubation media on the exchange-in kinetics of rhodopsin have been studied. We report here preliminary results of the apparent effect of phosphate ions on the incorporation of tritium into rhodopsin. The effect of solubilisation by detergent on the exchange-in kinetics of rhodopsin was also investigated.

A recent report on the hydrogen–tritium exchange of rod outer segment membranes [3] resulted in the suggestion that a major part of rhodopsin's polypeptide chain was freely accessible to the solvent. Our results indicate that the above suggestion may not be correct as complete tritiation of rhodopsin's labile hydrogens, a crucial point of their argument, appears not to have been achieved.

2. Materials and methods

All experiments were carried out in the dark or under dim red light.

2.1. *Preparation of rod outer segment membranes and rhodopsin*

Rod outer segment (ROS) membranes were prepared as previously described [5]. When used for hydrogen–tritium exchange experiments they were sonicated (MSE 150 W Ultrasonic disintegrator MK.2, 12 μm peak to peak) at 0°C, 5 times 15 sec with 30 sec between each burst. They were either used fresh or frozen in the presence of 5% w/w sucrose in isotonic phosphate buffer, pH 7, and then stored in the dark at –28°C for not more than 3 weeks.

Rhodopsin was purified in Ammonyx LO by a technique, inspired by that described by Applebury [6] and modified by Sardet, which will be described in detail elsewhere [7]. Rhodopsin was concentrated either by a hydroxyapatite column, rhodopsin being eluted with 150 mM P_i or by a column of Sepharose-Con A (Pharmacia, Uppsala, Sweden). In this case Ca^{2+} 1 mM and Mn^{2+} 1 mM were added to the rhodopsin solution before application to the Sepharose-Con A column. Rhodopsin was eluted with α-methyl-D-mannoside, 0.3 M and *d* methyl-D-glucoside, 0.3 M.

2.2. *Hydrogen–tritium exchange*

Rhodopsin's labile hydrogens were tritiated (exchange-in) by incubation of ROS membranes (5 mg rhodopsin/ml) or rhodopsin (2 mg/ml) in Ammonyx LO with tritiated water (2 mCi/ml) at 26°C. Exchange-in was carried out in three different buffers: (a) 10 mM immidazole-HCl, pH 7, with 100 mM NaCl, 1 mM DTT, 0.02% NaN_3 (Imm-NaCl buffer); (b) 10 mM immidazole, pH 7, with 150 mM P_i, 1 mM DTT, 0.02% NaN_3 (Imm-P_i buffer); (c) isotonic phosphate buffer, pH 7. For purified rhodopsin 0.5% Ammonyx LO was added to the buffer.

After the desired incubation time, exchange-out was initiated by removal of free tritium. Unless otherwise specified, exchange-out conditions were such that freely solvent accessible peptide hydrogens exchanged 126 times slower than under the exchange-in conditions; at 4°C in 50 mM maleate-NaOH buffer at pH 6 with 50 mM NaCl, 0.02% NaN_3 (exchange-out buffer). 0.5% Ammonyx LO was added to the buffer where necessary.

When exchange-out was to be studied for periods of less than one hour, the exchange-out was initiated by passing the sample through a gel filtration [8] column (Sephadex G-50, Pharmacia, or Ultrogel AcA 54 LKB). Aliquots (0.2 ml) of the eluant from this first column were withdrawn at intervals and the tritium lost by the rhodopsin removed by filtration through a second column. Determination of the concentration of rhodopsin and the associated radioactivity in the eluant permitted the number of unexchanged tritium atoms per rhodopsin molecule, at the moment of separation on the second filtration column, to be calculated [8]. For exchange-out periods of greater than an hour, exchange-out was initiated by diluting the sample three times with cold exchange-out buffer. It was then transfered to a rapid dialysis apparatus [9]. The dialysate was changed frequently. Aliquots (100 μl) of the dialysand were taken at intervals for determination of the number of unexchanged tritium atoms per rhodopsin molecule.

Rhodopsin concentrations were measured spectrophotometrically (ϵ_{500} = 40 000 M^{-1} cm^{-1}) [6]. ROS membranes being dissolved in 1% Ammonyx LO. Residual radioactivity associated with rhodopsin was measured in a Intertechnique scintillation counter.

3. Results

The general allure of the exchange-out kinetics of rhodopsin between one minute and one hour was investigated under a number of different conditions, amongst which were the conditions used by Downer and Englander [4]. Fig.1 shows our results which are in agreement with their exchange-out kinetics for unbleached ROS membranes. We observed however that these conditions do not correpond to a saturation of the exchange-in of tritium and that in conditions of more extensive exchange-in the number of slowly exchanging tritium atoms could be significantly increased.

In order to study the effect of the incubation medium on the amount of exchange-in, ROS membranes and rhodopsin in Ammonyx LO were incubated in two different buffers for various periods. The number of unexchanged tritium atoms per rhodopsin molecule after 20 min of exchange-out was then measured for each exchange-in time. The exchange-out time was fixed at 20 min because the exchange-out curve is then sufficently flat for an error of ± 1 min to be unimportant. The results are shown in fig.2. Three major points should be noted: (a) for the same incubation conditions the amount of tritium exchanged into membrane-bound or detergent-associated rhodopsin is almost the same; (b) the number of unexchanged tritium atoms (after 20 min of exchange-out) per rhodopsin molecule (membrane-bound or detergent-solubilised) can be increased by 50% when the incubation is carried out in Imm-P_i buffer instead of Imm-NaCl bufer; (c) in all cases a plateau in the exchange-in is reached.

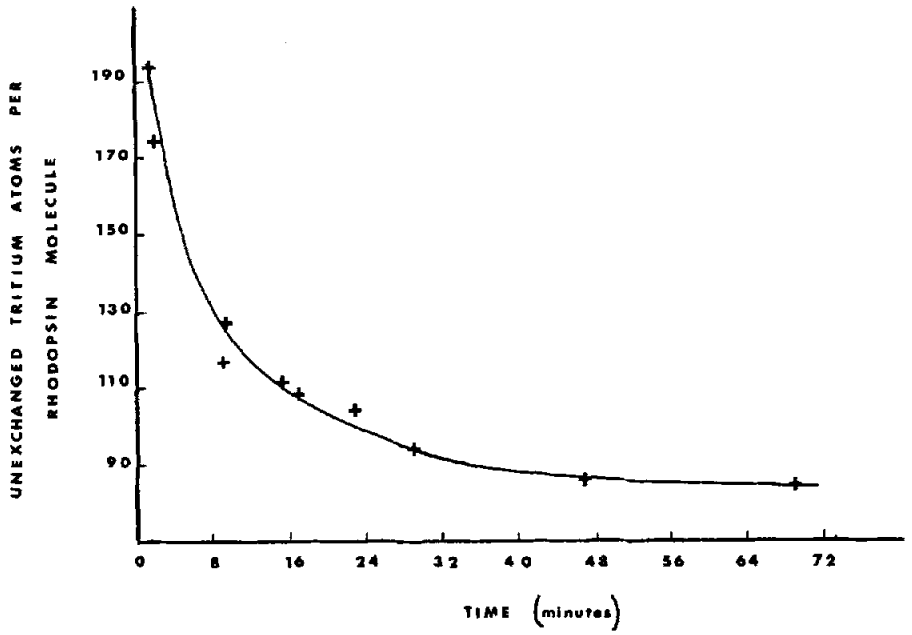

Fig.1. Exchange-out of tritium-labelled membrane-bound rhodopsin in 50 mM maleate-NaOH pH 5.5, 3°C. Exchange-in was performed in Ringer at pH 7.7, 3°C.

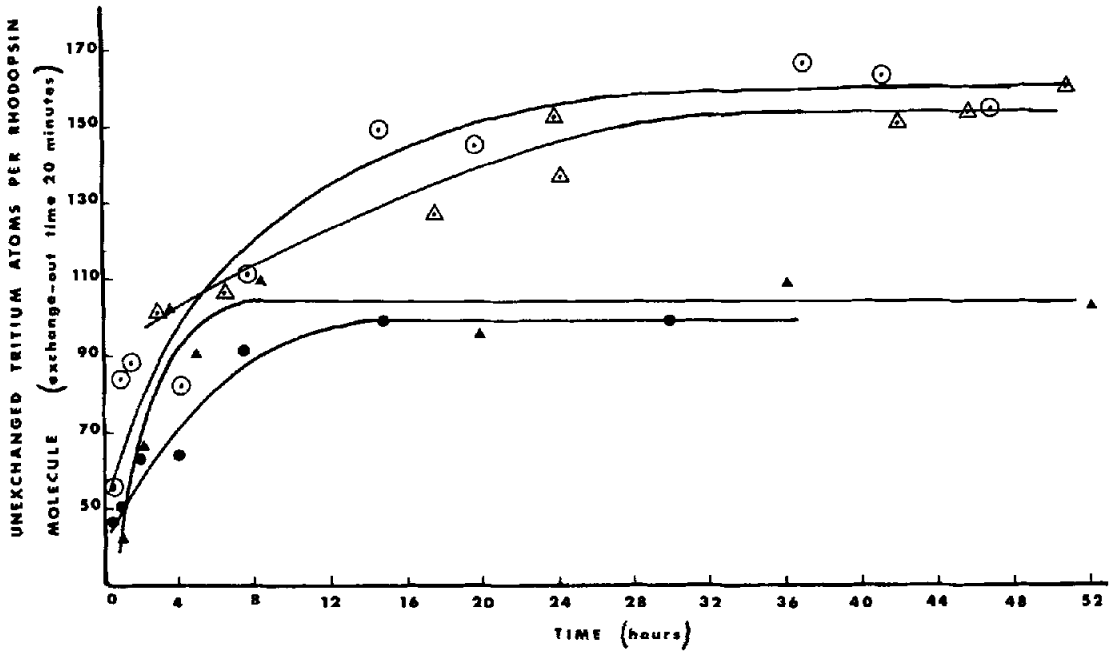

Fig.2. Effect of exchange-in conditions on tritium incorporation into rhodopsin. The exchange-out time was fixed at 20 min in exchange-out buffer at 4°C with 0.5% Ammonyx LO where necessary. Exchange-in was performed for various times. Symbols: (▲) ROS membranes in Imm-NaCl buffer; (●) Rhodopsin in 0.5% Ammonyx LO in Imm-NaCl buffer; (◬) ROS membranes in phosphate buffer; (⊙) Rhodopsin in 0.5% Ammonyx LO in Imm-P_i buffer.

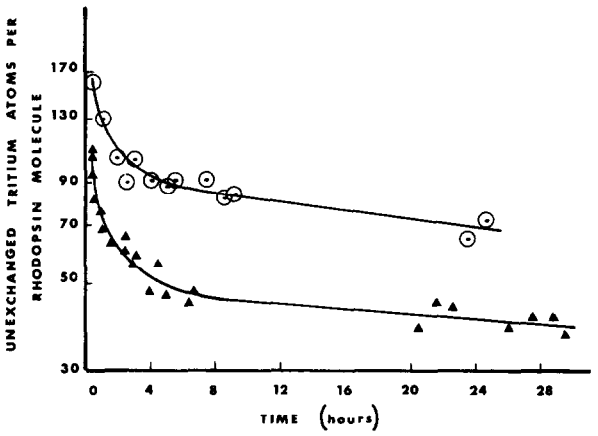

Fig.3. Exchange-out of tritium-labelled rhodopsin in exchange-out buffer at 4°C. Exchange-in time was sufficient to ensure that the plateau of incorporation was attained. Symbols are as in fig.2.

The kinetics of exchange-out of these additional slow tritium atoms was studied by observing the exchange-out of rhodopsin for up to 30 h. The samples were incubated for sufficient time for the exchange-in to have attained the plateau. The results shown in fig.3 are for the incubation conditions which differ the most (rhodopsin in Ammonyx LO Imm-P_i buffer and ROS membranes in Imm-NaCl buffer). The exchange-out kinetics, during the observed period, are similar for the two incubation conditions. In fact the $T_{1/2}$ of the tritium atoms which exchange after 8 h of exchange-out are almost the same for the two curves.

4. Discussion

We have shown that both membrane-bound and detergent-solubilised rhodopsin posses an important number of very slowly exchanging hydrogen atoms. The number of slowly exchange tritium atoms incorporated into rhodopsin is greatly increased by the presence of phosphate ions in the incubation medium.

Two artifacts could be responsible for this observed additional exchange-in of tritium atoms. (a) A slow thermal bleaching of rhodopsin in the presence of phosphate, would increase the calculated number of rhodopsin associated tritium atoms. This was excluded by spectrophotometric controls being made on the samples of long exchange-in times. (b) A non-bleaching denaturation of part of the rhodopsin molecule would increase the solvent accessibility of the protein and hence the rate of hydrogen exchange. However in this case, the rate of exchange-out of the additional tritium atoms should be fast. Even perhaps, depending on the degree of denaturation, at a rate approaching that of a freely accessible polypeptide chain ($T_{1/2}$ = 7.6 sec for the peptide hydrogens of poly-D,L-alanine at pH 6, 4°C) [2]. The fact that the additional tritium atoms exchange-out with a $T_{1/2}$ of about 55 to 60 h, which is similar to that of the slowest observed class of tritium atoms of rhodopsin incubated in Imm-NaCl buffer, indicates that this hypothesis can be excluded.

It can also be concluded that no major conformational change or denaturation of the rhodopsin occured when it was removed from the membrane since the observed exchange-in and exchange-out kinetics of ROS membranes and of rhodopsin in Ammonyx LO are very similar. We have results however, that indicate that this is no longer the case when bleached rhodopsin is considered [10].

Our results indicate that the conclusions drawn by Downer and Englander [4] about the structure of rhodopsin may not be correct since complete tritium exchange-in appears not to have occured. Complete exchange-in was assumed because (a) it is generaly accepted that all amino acid side chain hydrogens exchange too fast to be observed [2]; and (b) the number of unexchanged hydrogen atoms per peptide group extrapolated to zero exchange-out time and the number of peptide and primary amide hydrogens per peptide group calculated from the amino acid composition were almost the same. However the long held premise that side chain hydrogens are not observable may not be true. Indeed in cytochrome *c* [3] there are twice as many non-peptide hydrogens unexchanged after 24 h of exchange-out at 20°C, pH 7 than peptide hydrogens. That complete exchange-in may not have occured is substantiated by the difference that they observe between bleached frog and cattle rhodopsin (a decrease in frog and an increase in cattle of the number of unexchanged hydrogen atoms upon bleaching). The cattle rhodopsin however was bleached in conditions that

would have increased the amount of exchange-in (warming the sample to 37°C during illumination).

The way in which the increased exchange-in is produced by phosphate ions or whether phosphate ions are directly responsible is not yet clear. If phosphate ions had induced a conformational change in rhodopsin then it must be reversible. ROS membranes are purified in phosphate buffer and during purification rhodopsin is eluted from the hydroxyapatite column by phosphate ions. The increase in tritium exchange-in is however real and is not affected by removal of rhodopsin from the membrane. Also these additional tritium atoms are incorporated into a region of rhodopsin of low solvent accessibility.

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